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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 6 :</b> <b>G01N 33/574, 33/48, C12Q 1/68</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/54738</b> <b>(43) International Publication Date:</b> 28 October 1999 (28.10.99)
<b>(21) International Application Number:</b> PCT/US99/05766 <b>(22) International Filing Date:</b> 16 March 1999 (16.03.99)  <b>(30) Priority Data:</b> 09/061,709 17 April 1998 (17.04.98) US  <b>(71) Applicant:</b> LUDWIG INSTITUTE FOR CANCER RE- SEARCH [CH/US]; 605 Third Avenue, New York, NY 10103 (US).  <b>(72) Inventors:</b> CHEN, Yao-Tseng; 525 East 68 Street, New York, NY 10021 (US). GURE, Ali; 1275 York Avenue, New York, NY 10021 (US). TSANG, Solam; 1275 York Avenue, New York, NY 10021 (US). STOCKERT, Elisabeth; 1275 York Avenue, New York, NY 10002 (US). JAGER, Elke; Steinbacher Hohl 2-28, D-60488 Frankfurt am Main (DE). KNUTH, Alexander; Steinbacher Hohl 2-28, D-60488 Frankfurt am Main (DE). OLD, Lloyd, J.; 605 Third Avenue, New York, NY 10021 (US).  <b>(74) Agent:</b> HANSON, Norman, D.; Fulbright & Jaworski LLP, 666 Fifth Avenue, New York, NY 10103 (US).		<b>(81) Designated States:</b> AU, CA, CN, JP, KR, NZ, ZA, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> ISOLATED NUCLEIC ACID MOLECULE ENCODING CANCER ASSOCIATED ANTIGENS, THE ANTIGENS PER SE, AND USES THEREOF  <b>(57) Abstract</b>  The invention relates to newly identified cancer associated antigens, referred to as CT7, KOC-2 and KOC-3. The invention also relates to observations regarding known molecule KOC-1. It has been discovered that each of these molecules provokes antibodies when expressed by a subject. The ramifications of this observation are also a part of this invention.		

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ISOLATED NUCLEIC ACID MOLECULE ENCODING  
CANCER ASSOCIATED ANTIGENS, THE ANTIGENS PER SE,  
AND USES THEREOF

**RELATED APPLICATION**

This application is a continuation in part of Serial No. 09/061,709 filed April 17, 1998, incorporated by reference.

**FIELD OF THE INVENTION**

This invention relates to antigens associated with cancer, the nucleic acid molecules encoding them, as well as the uses of these.

**BACKGROUND AND PRIOR ART**

It is fairly well established that many pathological conditions, such as infections, cancer, autoimmune disorders, etc., are characterized by the inappropriate expression of certain molecules. These molecules thus serve as "markers" for a particular pathological or abnormal condition. Apart from their use as diagnostic "targets", i.e., materials to be identified to diagnose these abnormal conditions, the molecules serve as reagents which can be used to generate diagnostic and/or therapeutic agents. A by no means limiting example of this is the use of cancer markers to produce antibodies specific to a particular marker. Yet another non-limiting example is the use of a peptide which complexes with an MHC molecule, to generate cytolytic T cells against abnormal cells.

Preparation of such materials, of course, presupposes a source of the reagents used to generate these. Purification from cells is one laborious, far from sure method of doing so. Another preferred method is the isolation of nucleic acid molecules which encode a particular marker, followed by the use of the isolated encoding molecule to express the desired molecule.

Two basic strategies have been employed for the detection of such antigens, in e.g., human tumors. These will be referred to as the genetic approach and the biochemical approach. The genetic approach is exemplified by, e.g., dePlaen et al., Proc. Natl. Sci. USA 85: 2275

(1988), incorporated by reference. In this approach, several hundred pools of plasmids of a cDNA library obtained from a tumor are transfected into recipient cells, such as COS cells, or into antigen-negative variants of tumor cell lines which are tested for the expression of the specific antigen. The biochemical approach, exemplified by, e.g., O. Mandelboim, et al., *Nature* 369: 69 (1994) incorporated by reference, is based on acidic elution of peptides which have bound to MHC-class I molecules of tumor cells, followed by reversed-phase high performance liquid chromatography (HPLC). Antigenic peptides are identified after they bind to empty MHC-class I molecules of mutant cell lines, defective in antigen processing, and induce specific reactions with cytotoxic T-lymphocytes. These reactions include induction of CTL proliferation, TNF release, and lysis of target cells, measurable in an MTT assay, or a  $^{51}\text{Cr}$  release assay.

These two approaches to the molecular definition of antigens have the following disadvantages: first, they are enormously cumbersome, time-consuming and expensive; and second, they depend on the establishment of cytotoxic T cell lines (CTLs) with predefined specificity.

The problems inherent to the two known approaches for the identification and molecular definition of antigens is best demonstrated by the fact that both methods have, so far, succeeded in defining only very few new antigens in human tumors. See, e.g., van der Bruggen et al., *Science* 254: 1643-1647 (1991); Brichard et al., *J. Exp. Med.* 178: 489-495 (1993); Coulie, et al., *J. Exp. Med.* 180: 35-42 (1994); Kawakami, et al., *Proc. Natl. Acad. Sci. USA* 91: 3515-3519 (1994).

Further, the methodologies described rely on the availability of established, permanent cell lines of the cancer type under consideration. It is very difficult to establish cell lines from certain cancer types, as is shown by, e.g., Oettgen, et al., *Immunol. Allerg. Clin. North. Am.*

10: 607-637 (1990). It is also known that some epithelial cell type cancers are poorly susceptible to CTLs in vitro, precluding routine analysis. These problems have stimulated the art to develop additional methodologies for identifying cancer associated antigens.

One key methodology is described by Sahin, et al., Proc. Natl. Acad. Sci. USA 92: 11810-11913 (1995), incorporated by reference. Also, see U.S. Patent No. 5,698,396, and Application Serial No. 08/479,328, filed on June 7, 1995 and January 3, 1996, respectively. All three of these references are incorporated by reference. To summarize, the method involves the expression of cDNA libraries in a prokaryotic host. (The libraries are secured from a tumor sample). The expressed libraries are then immunoscreened with absorbed and diluted sera, in order to detect those antigens which elicit high titer humoral responses. This methodology is known as the SEREX method ("Serological identification of antigens by Recombinant Expression Cloning"). The methodology has been employed to confirm expression of previously identified tumor associated antigens, as well as to detect new ones. See the above referenced patent applications and Sahin, et al., supra, as well as Crew, et al., EMBO J 144: 2333-2340 (1995).

This methodology has been applied to a range of tumor types, including those described by Sahin et al., supra, and Pfreundschuh, supra, as well as to esophageal cancer (Chen et al., Proc. Natl. Acad. Sci. USA 94: 1914-1918 (1997)); lung cancer (Güre et al., Cancer Res. 58: 1034-1041 (1998)); colon cancer (Serial No. 08/948,705 filed October 10, 1997) incorporated by reference, and so forth. Among the antigens identified via SEREX are the SSX2 molecule (Sahin et al., Proc. Natl. Acad. Sci. USA 92: 11810-11813 (1995); Tureci et al., Cancer Res. 56: 4766-4772 (1996); NY-ESO-1 Chen, et al., Proc. Natl. Acad. Sci. USA 94: 1914-1918 (1997); and SCP1 (Serial No. 08/892,705 filed July 15, 1997) incorporated by reference.

Analysis of SEREX identified antigens has shown overlap between SEREX defined and CTL defined antigens. MAGE-1, tyrosinase, and NY-ESO-1 have all been shown to be recognized by patient antibodies as well as CTLs, showing that humoral and cell mediated responses do act in concert.

It is clear from this summary that identification of relevant antigens via SEREX is a desirable aim. The inventors have modified standard SEREX protocols and have screened a cell line known to be a good source of the antigens listed supra, using allogeneic patient sample. New antigens have been identified in this way and have been studied. Also, a previously known molecule has now been identified via SEREX techniques.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

##### Example 1

The melanoma cell referred to as SK-MEL-37 was used, because it has been shown to express a number of members of the CT antigen family, including MAGE-1 (Chen et al., Proc. Natl. Acad. Sci. USA 91: 1004-1008 (1994); NY-ESO-1 (Chen et al. Proc. Natl. Acad. Sci. USA 94: 1914-1918 (1997)); and various members of the SSX family (Gure et al., Int. J. Cancer 72: 965-971 (1997)).

Total RNA was extracted from cultured samples of SK-MEL-37 using standard methods, and this was then used to construct a cDNA library in commercially available, λZAP expression vector, following protocols provided by the manufacturer. The cDNA was then transfected into E. coli and screened, following Sahin et al., Proc. Natl. Acad. Sci. USA 92: 11810-11813 (1995), incorporated by reference, and Pfreundschuh, U.S. Patent No. 5,698,396, also incorporated by reference. The screening was done with allogeneic patient serum "NW38." This serum had been shown, previously, to contain high titer antibodies against MAGE-1 and NY-

ESO-1. See, e.g., Jäger et al., J. Exp. Med. 187: 265-270 (1998), incorporated by reference.

In brief, serum was diluted 1:10, preabsorbed with lysates of transfected E. coli, further diluted to 1:2000, and then incubated overnight at room temperature with nitrocellulose membranes containing phage plaques, prepared in accordance with Sahin et al., and Pfreundschuh, supra. The library contained a total of  $2.3 \times 10^7$  primary clones. After washing, the filters were incubated with alkaline phosphatase conjugated, goat anti-human Fc $\gamma$  secondary antibodies, and were then visualized by incubating with 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium.

After screening  $1.5 \times 10^5$  of the clones, a total of sixty-one positives had been identified. Given this number, screening was stopped, and the positive clones were subjected to further analysis.

#### Example 2

The positive clones identified in example 1, supra, were purified, the inserts were excised in vitro, and inserted into a commercially available plasmid, pBK-CMV, and then evaluated on the basis of restriction mapping with EcoRI and XbaI. Clones which represented different inserts on the basis of this step were sequenced, using standard methodologies.

There was a group of 10 clones, which could not be classified other than as "miscellaneous genes", in that they did not seem to belong to any particular family. They consisted of 9 distinct genes, of which four were known, and five were new. The fifty one remaining clones were classified into four groups. The data are presented in Tables 1 and 2, which follow.

The largest group are genes related to KOC ("KH-domain containing gene, overexpressed in cancer" which has been shown to be overexpressed in pancreatic cancer, and maps to



chromosome 7p11.5. See Müller-Pillasch et al., *Oncogene* 14: 2729-2733 (1997). Two of the 33 were derived from the KOC gene, and the other 31 were derived from two previously unidentified, but related genes. Examples 6 et seq. describe work on this group of clones.

Eleven clones, i.e., Group 2, were MAGE sequences. Four were derived from MAGE-4a, taught by DePlaen et al., *Immunogenetics* 40: 360-369, Genbank U10687, while the other 7 hybridized to a MAGE-4a probe, derived from the 5' sequence, suggesting they belong to the MAGE family.

The third group consisted of five clones of the NY-ESO-1 family. Two were identical to the gene described by Chen et al., *Proc. Natl. Acad. Sci. USA* 94: 1914-1918 (1997), and in Serial No. 08/725,182, filed October 3, 1996, incorporated by reference. The other three were derived from a second member of the NY-ESO-1 family, i.e., LAGE-1. See U.S. application Serial No. 08/791,495, filed January 27, 1997 and incorporated by reference.

The fourth, and final group, related to a novel gene referred to as CT7. This gene, the sequence of which is presented as SEQ ID NO: 1, was studied further.

Table 1. SEREX-identified genes from allogeneic screening of SK-MEL-37 library

Gene group	# of clones	Comments
KOC	33	derived from 3 related genes
MAGE	11	predominantly MAGE-4a (see text)
NY-ESO-1	5	derived from 2 related genes (NY-ESO-1, LAGE-1)
CT7	2	new cancer/testis antigen
Miscellaneous	10	see Table 2

Table 2. SEREX-identified genes from allogeneic screening of SK-MEL-37 library--  
Miscellaneous group

Clone designation	Gene
MNW-4, MNW-7	S-adenyl homocysteine hydrolase
MNE-6a	Glutathione synthetase
MNW-24	proliferation-associated protein p38-2G4
MNW-27a	phosphoribosyl pyrophosphate synthetase-associated protein 39
MNW-6b	unknown gene, identical to sequence tags from pancreas, uterus etc.
MNW-14b	unknown gene, identical to sequence tags from lung, brain, fibroblast etc.
MNW-34a	unknown gene, identical to sequence tags from multiple tissues
MNW-17	unknown gene, identical to sequence tags from pancreas and fetus
MNW-29a	unknown gene, no significant sequence homology, universally expressed

### Example 3

The two clones for CT7, referred to supra, were 2184 and 1965 base pairs long. Analysis of the longer one was carried out. It presented an open reading frame of 543 amino acids, which extended to the 5' end of the sequence, indicating that it was a partial cDNA clone.

In order to identify the complete sequence, and to try to identify additional, related genes, a human testicular cDNA library was prepared, following standard methods, and screened with probes derived from the longer sequence, following standard methods.

Eleven positives were detected, and sequenced, and it was found that all derived from the same gene. When the polyA tail was excluded, full length transcript, as per SEQ ID NO: 1, consisted of 4265 nucleotides, broken down into 286 base pairs of untranslated 5' - region, a coding region of 3429 base pairs, and 550 base pairs of untranslated 3' region. The predicted protein is 1142 amino acids long, and has a calculated molecular mass of about 125 kilodaltons. See SEQ ID NO: 2.

The nucleotide and deduced amino acid sequences were screened against known databases, and there was some homology with the MAGE-10 gene, described by DePlaen et al., Immunogenetics 40: 360-369 (1994). The homology was limited to about 210 carboxy terminal amino acids, i.e., amino acids 908-1115 of the subject sequence, and 134-342 of MAGE-10. The percent homology was 56%, rising to 75% when conservative changes are included.

There was also extensive homology with a sequence reported by Lucas et al., Canc. Res. 58: 743-752 (1998), and application Serial No. 08/845,528 filed April 25, 1997, also incorporated by reference. A total of 14 nucleotides differ in the open reading frame, resulting in a total of 11 amino acids which differ between the sequences.

The 5' region of the nucleotide and sequence and corresponding amino acid sequence demonstrates a strikingly repetitive pattern, with repeats rich in serine, proline, glutamine, and leucine, with an almost invariable core of PQSPLQI (SEQ ID NO: 3). In the middle of the molecule, 11 almost exact repeats of 35 amino acids were observed. The repetitive portions make up about 70% of the entire sequence, begin shortly after translation initiation, at position 15, and ending shortly before the region homologous to MAGE 4a.

#### Example 4

The expression pattern for mRNA of CT7 was then studied, in both normal and malignant tissues. RT-PCR was used, employing primers specific for the gene. The estimated melting temperature of the primers was 65-70°C, and they were designed to amplify 300-600 base pair segments. A total of 35 amplification cycles were carried out, at an annealing temperature of 60°C. Table 3, which follows, presents the data for human tumor tissues. CT7 was expressed in a number of different samples. Of fourteen normal tissues tested, there was strong expression in testis, and none in colon, brain, adrenal, lung, breast, pancreas, prostate, thymus or uterus tissue. There was low level expression in liver, kidney, placenta and fetal brain, with fetal brain showing three transcripts of different size. The level of expression was at least 20-50 times lower than in testis. Melanoma cell lines were also screened. Of these 7 of the 12 tested showed strong expression, and one showed weak expression.

Table 3. CT7 mRNA expression in various human tumors by RT-PCR

Tumor type	mRNA, positive/total
Melanoma	7/10
Breast cancer	3/10
Lung cancer	3/9
Head/neck cancer	5/14
Bladder cancer	4/9
Colon cancer	1/10
Leimyosarcoma	1/4
synovial sarcoma	2/4
Total	26/70

#### Example 5

Southern blotting experiments were then carried out to determine if CT7 belonged to a family of genes. In these experiments, genomic DNA was extracted from normal human tissues. It was digested with BamHI, EcoRI, and HindIII, separated on a 0.7% agarose gel, blotted onto a nitrocellulose filter, and hybridized, at high stringency (65°C, aqueous buffer), with a <sup>32</sup>P labelled probe, derived from SEQ ID NO: 1.

The blotting showed anywhere from two to four bands, suggesting one or two genes in the family.

#### Example 6

As noted in example 2, supra, thirty three of the sixty one positive clones were related to KOC. Clones were sequenced using standard methodologies. As indicated supra, one clone

was identical to KOC, initially reported by Müller-Pillasch, et al., supra. Given that two additional related sequences were identified, the known KOC gene is referred to as KOC-1 hereafter (SEQ ID NO: 4). The second clone, referred to as KOC-2 hereafter, was found once. The sequence is presented as SEQ ID NO: 5. Its deduced amino acid sequence is 72.5% identical to that for KOC-1.

The third sequence, KOC-3, appeared thirty times (SEQ ID NO: 6). Its deduced amino acid sequence is 63% identical to KOC-1.

Testicular cDNA libraries were analyzed in the same way that the SK-MEL-37 library was analyzed, i.e., with allogeneic serum from NW-38. See example 3, supra.

Following analysis of testicular libraries, a longer form of KOC-2 was isolated. This is presented as SEQ ID NO: 7. When SEQ ID NOS: 5 & 7 are compared, the former is 1705 base pairs in length, without a polyA tail. It contains 1362 base pairs of coding sequence, and 343 base pairs of 3' untranslated sequence. Nucleotides 275-1942 of SEQ ID NO: 7 are identical to nucleotides 38-1705 of SEQ ID NO: 5.

The sequence of KOC-3, set forth as SEQ ID NO: 6, is 3412 base pairs long, and consists of 72 base pairs of 5' untranslated region, 1707 base pairs of open reading frame, and 1543 base pairs of untranslated, 3' region. An alternate form was also isolated, (SEQ ID NO: 8), and is 129 base pairs shorter than SEQ ID NO: 6.

#### Example 7

Expression patterns for KOC-1, KOC-2 and KOC-3 were then studied, using RT-PCR and the following primer pairs:

GAAAGTATCT TCAAGGACGC C

CTGCAAGGGG TTTTGCTGGG CG

(SEQ ID NOS: 9 & 10).

TCCTTGCGCG CTGCGGCCTC AG

CCAACTGGTG GCCATTCAGCT TC

(SEQ ID NOS: 11 & 12)

GCTCTTTGGG GACAGGAAGG TC

GACGTTGACA ACGGCGGTTT CT

(SEQ ID NOS: 13 & 14).

SEQ ID NOS: 9 & 10 were designed to amplify KOC-1 while SEQ ID NOS: 11 & 12 were designed to amplify KOC-2, and SEQ ID NOS: 13 & 14 were designed to amplify KOC-3.

To carry out the RT-PCR, relevant primer pairs were added to cDNA samples prepared from various mRNAs by reverse transcription. PCR was then carried out at an annealing temperature of 60°C, and extension at 72°C, for 35 cycles. The resulting products were then analyzed by gel electrophoresis.

SEQ ID NOS 9 & 10 amplify nucleotides 305-748 of SEQ ID NO: 1. A variety of normal and malignant cell types were tested. Strong expression was found in testis, moderate expression in normal brain, and low levels of expression were found in normal colon, kidney, and liver.

The Müller-Pillasch paper, cited supra, identified expression of KOC-1 in pancreatic tumor cell lines, gastric cancer, and normal placenta, via Northern blotting. This paper also reported that normal heart, brain, lung, liver, kidney and pancreatic tissue were negative for

KOC-1 expression. The difference in results suggests that the level of expression of KOC-1 is very low in normal tissues.

When KOC-2 expression was studied, the only positive normal tissue was testis (brain, liver, kidney and colon were negative).

Modification of the protocol for detecting KOC-2 resulted in positives in normal kidney, liver and melanoma.

When KOC-3 expression was studied, it was found that the gene was universally expressed in normal tissues, with highest expression in testis.

The pattern of expression of KOC-3 in different melanoma cell lines was analyzed, using standard Northern blotting. Over expression in several cell lines was observed, which is consistent with the more frequent isolation of this clone than any other.

#### Example 8

A study was carried out to determine if KOC-1 is expressed at higher levels in melanoma cells, as compared to normal skin cells. This was done using representational difference analysis, or "RDA." See Lisitsyn, et al. Science 259: 946-951 (1993), and O'Neill, et al. Nucl. Acids Res. 25:2681-2 (1997), both of which are incorporated by reference. Specifically, tester cDNA was taken from SK-MEL-37, and driver cDNA was taken from a skin sample representing mRNA from various cell types in the skin. The cDNAs were digested with either Tsp509I, Hsp92II, or DpnII. When DpnII was the enzyme used for digestion, adaptor oligonucleotides R-Bgl-24, J-Bgl-24, and N-Bgl-24 described by O'Neill, et al., supra, and Hubank, et al. Nucl. Acids Res.



22:5640-5648 (1994) were used. When Tsp509I was the endonuclease, the same adaptors were used, as were R-Tsp-12, i.e.:

AATTTGCGGT GA

(SEQ ID NO: 15)

J-Tsp-12, i.e.:

AATTTGTTCA TG

(SEQ ID NO: 16)

and N-Tsp-12, i.e.:

AATTTTCCCT CG

(SEQ ID NO: 17)

When Hsp92II was the endonuclease, the adaptors were:

R-Hsp-24, i.e.:

AGCACTCTCC AGCCTCTCAC CATG

(SEQ ID NO: 18);

J-Hsp-24, i.e.:

ACCGACGTCG ACTATCATG CATG

(SEQ ID NO: 19);

N-Hsp-24, i.e.:

AGGCAACTGT GCTATCCGAG CATG

(SEQ ID NO: 20);

R-Hsp-8, i.e.:

GTGAGAGG

(SEQ ID NO: 21);

J-Hsp-8, i.e.:

CATGGATG

(SEQ ID NO: 22);

N-Hsp-8, i.e.:

CTCGGATA

(SEQ ID NO: 23).

In order to hybridize tester and driver, either 3XEE buffer (30mM EPPS, pH8, 3mM EDTA), or a buffer of 2.4M tetraethylammonium chloride (TEACl) 3mM EDTA, 10mM Tris HCl, pH8, was used. When DNA was dissolved in 10  $\mu$ l of TEACl buffer, it was denatured at 80°C for 10 minutes, followed by renaturing at 42°C for 20 hours. Amplicons were gel purified, and the DP3 or DP2 product was ligated into BamHI (when DpnII was used), EcoRI (when Tsp 509I was used), or SphI (when Hsp92II was used), cloning vectors were digested, and then sequenced. Sequence analysis of the cDNA molecules derived from these experiments identified KOC-1 as one of the genes isolated, indicating that KOC-1 mRNA is present at a higher level in Sk-Mel 37 cells as compared to normal skin cells.

The foregoing examples describe the isolation of a nucleic acid molecule which encodes a cancer associated antigen. "Associated" is used herein because while it is clear that the relevant molecule was expressed by several types of cancer, other cancers, not screened herein, may also express the antigen.

The invention relates to those nucleic acid molecules which encode the antigens CT7, KOC-2 and KOC-3, as described herein, such as a nucleic acid molecule consisting of the nucleotide sequence SEQ ID NO: 1, molecules comprising the nucleotide sequence of SEQ ID

NO: 5, 6, 7 or 8 and so forth. Also embraced are those molecules which are not identical to SEQ ID NOS: 1, 5, 6, 7 or 8, but which encode the same antigen.

Also a part of the invention are expression vectors which incorporate the nucleic acid molecules of the invention, in operable linkage (i.e., "operably linked") to a promoter. Construction of such vectors, such as viral (e.g., adenovirus or Vaccinia virus) or attenuated viral vectors is well within the skill of the art, as is the transformation or transfection of cells, to produce eukaryotic cell lines, or prokaryotic cell strains which encode the molecule of interest. Exemplary of the host cells which can be employed in this fashion are COS cells, CHO cells, yeast cells, insect cells (e.g., *Spodoptera frugiperda*), NIH 3T3 cells, and so forth. Prokaryotic cells, such as *E. coli* and other bacteria may also be used. Any of these cells can also be transformed or transfected with further nucleic acid molecules, such as those encoding cytokines, e.g., interleukins such as IL-2, 4, 6, or 12 or HLA or MHC molecules.

Also a part of the invention are the antigens described herein, both in original form and in any different post translational modified forms. The molecules are large enough to be antigenic without any posttranslational modification, and hence are useful as immunogens, when combined with an adjuvant (or without it), in both precursor and post-translationally modified forms. Antibodies produced using these antigens, both poly and monoclonal, are also a part of the invention as well as hybridomas which make monoclonal antibodies to the antigens. The whole protein can be used therapeutically, or in portions, as discussed infra. Also a part of the invention are antibodies against this antigen, be these polyclonal, monoclonal, reactive fragments, such as Fab, (F(ab)<sub>2</sub>)' and other fragments, as well as chimeras, humanized antibodies, recombinantly produced antibodies, and so forth.

As is clear from the disclosure, one may use the proteins and nucleic acid molecules of the invention diagnostically. The SEREX methodology discussed herein is premised on an immune response to a pathology associated antigen. Hence, one may assay for the relevant pathology via, e.g., testing a body fluid sample of a subject, such as serum, for reactivity with the antigen *per se*. Reactivity would be deemed indicative of possible presence of the pathology. So, too, could one assay for the expression of any of the antigens via any of the standard nucleic acid hybridization assays which are well known to the art, and need not be elaborated upon herein. One could assay for antibodies against the subject molecules, using standard immunoassays as well.

Analysis of SEQ ID NO: 1, 5, 6, 7 and 8 will show that there are 5' and 3' non-coding regions presented therein. The invention relates to those isolated nucleic acid molecules which contain at least the coding segment, i.e., nucleotides 54-593, of SEQ ID NO: 1, nucleotides 1-1019 of SEQ ID NO: 3, nucleotides 73-1780 of SEQ ID NO: 8, and so forth, and which may contain any or all of the non-coding 5' and 3' portions.

Also a part of the invention are portions of the relevant nucleic acid molecules which can be used, for example, as oligonucleotide primers and/or probes, such as one or more of SEQ ID NOS: 7, 8, 9, 10, 11, 12, 13 or 14 as well as amplification product like nucleic acid molecules comprising at least nucleotides 305-748 of SEQ ID NO: 1.

As was discussed supra, study of other members of the "CT" family reveals that these are also processed to peptides which provoke lysis by cytolytic T cells. There has been a great deal of work on motifs for various MHC or HLA molecules, which is applicable here. Hence, a further aspect of the invention is a therapeutic method, wherein one or more peptides derived

from the antigens of the invention which bind to an HLA molecule on the surface of a patient's tumor cells are administered to the patient, in an amount sufficient for the peptides to bind to the MHC/HLA molecules, and provoke lysis by T cells. Any combination of peptides may be used. These peptides, which may be used alone or in combination, as well as the entire protein or immunoreactive portions thereof, may be administered to a subject in need thereof, using any of the standard types of administration, such as intravenous, intradermal, subcutaneous, oral, rectal, and transdermal administration. Standard pharmaceutical carriers, adjuvants, such as saponins, GM-CSF, and interleukins and so forth may also be used. Further, these peptides and proteins may be formulated into vaccines with the listed material, as may dendritic cells, or other cells which present relevant MHC/peptide complexes.

Similarly, the invention contemplates therapies wherein nucleic acid molecules which encode the proteins of the invention, one or more or peptides which are derived from these proteins are incorporated into a vector, such as a Vaccinia or adenovirus based vector, to render it transfectable into eukaryotic cells, such as human cells. Similarly, nucleic acid molecules which encode one or more of the peptides may be incorporated into these vectors, which are then the major constituent of nucleic acid bases therapies.

Any of these assays can also be used in progression/regression studies. One can monitor the course of abnormality involving expression of these antigens simply by monitoring levels of the protein, its expression, antibodies against it and so forth using any or all of the methods set forth supra.

It should be clear that these methodologies may also be used to track the efficacy of a therapeutic regime. Essentially, one can take a baseline value for a protein of interest using any

of the assays discussed supra, administer a given therapeutic agent, and then monitor levels of the protein thereafter, observing changes in antigen levels as indicia of the efficacy of the regime.

As was indicated supra, the invention involves, inter alia, the recognition of an "integrated" immune response to the molecules of the invention. One ramification of this is the ability to monitor the course of cancer therapy. In this method, which is a part of the invention, a subject in need of the therapy receives a vaccination of a type described herein. Such a vaccination results, e.g., in a T cell response against cells presenting HLA/peptide complexes on their cells. The response also includes an antibody response, possibly a result of the release of antibody provoking proteins via the lysis of cells by the T cells. Hence, one can monitor the effect of a vaccine, by monitoring an antibody response. As is indicated, supra, an increase in antibody titer may be taken as an indicia of progress with a vaccine, and vice versa. Hence, a further aspect of the invention is a method for monitoring efficacy of a vaccine, following administration thereof, by determining levels of antibodies in the subject which are specific for the vaccine itself, or a large molecule of which the vaccine is a part.

The identification of the subject proteins as being implicated in pathological conditions such as cancer also suggests a number of therapeutic approaches in addition to those discussed supra. The experiments set forth supra establish that antibodies are produced in response to expression of the protein. Hence, a further embodiment of the invention is the treatment of conditions which are characterized by aberrant or abnormal levels of one or more of the proteins, via administration of antibodies, such as humanized antibodies, antibody fragments, and so forth. These may be tagged or labelled with appropriate cystostatic or cytotoxic reagents.

T cells may also be administered. It is to be noted that the T cells may be elicited in vitro using immune responsive cells such as dendritic cells, lymphocytes, or any other immune responsive cells, and then reperfused into the subject being treated.

Note that the generation of T cells and/or antibodies can also be accomplished by administering cells, preferably treated to be rendered non-proliferative, which present relevant T cell or B cell epitopes for response, such as the epitopes discussed supra.

The therapeutic approaches may also include antisense therapies, wherein an antisense molecule, preferably from 10 to 100 nucleotides in length, is administered to the subject either "nude" or in a carrier, such as a liposome, to facilitate incorporation into a cell, followed by inhibition of expression of the protein. Such antisense sequences may also be incorporated into appropriate vaccines, such as in viral vectors (e.g., Vaccinia), bacterial constructs, such as variants of the known BCG vaccine, and so forth.

Also a part of the inventions are Peptides, such as those set forth in Figure 1, and those which have as a core sequence

PQSPLQI (SEQ ID NO.: 3)

These peptides may be used therapeutically, via administration to a patient who expresses CT7 in connection with a pathology, as well as diagnostically, i.e., to determine if relevant antibodies are present and so forth.

Other features and applications of the invention will be clear to the skilled artisan, and need not be set forth herein. The terms and expression which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expression of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

**We claim:**

1. Isolated nucleic acid molecule which encodes a cancer associated antigen, whose amino acid sequence is identical to the amino sequence encoded by nucleotides 287 to 3714 of SEQ ID NO: 1.
2. The isolated nucleic acid molecule of claim 1, consisting of nucleotides 287-3714 of SEQ ID NO: 1.
3. The isolated nucleic acid molecule of claim 1, consisting of anywhere from nucleotide 1 through nucleotide 4265 of SEQ ID NO: 1, with the proviso that said isolated nucleic acid molecule contains at least nucleotides 287-3714 of SEQ ID NO: 1.
4. Expression vector comprising the isolated nucleic acid molecule of claim 1, operably linked to a promoter.
5. Expression vector comprising the isolated nucleic acid molecule of claim 3, operably linked to a promoter.
6. Eukaryotic cell line or prokaryotic cell strain, transformed or transfected with the expression vector of claim 4.
7. Eukaryotic cell line or prokaryotic cell strain, transformed or transfected with the expression vector of claim 5.
8. Isolated cancer associated antigen comprising all or part of the amino acid sequence encoded by nucleotides 287-3714 of SEQ ID NO: 1.
9. Eukaryotic cell line or prokaryote cell strain, transformed or transfected with the isolated nucleic acid molecule of claim 1.
10. The eukaryotic cell line of claim 9, wherein said cell line is also transfected with a nucleic acid molecule coding for a cytokine.



11. The eukaryotic cell line of claim 10, wherein said cell line is further transfected by a nucleic acid molecule coding for an HLA molecule.
12. The eukaryotic cell line of claim 10, wherein said cytokine is an interleukin.
13. The biologically pure culture of claim 12, wherein said interleukin is IL-2, IL-4 or IL-12.
14. The eukaryotic cell line of claim 9, wherein said cell line has been rendered non-proliferative.
15. The eukaryotic cell line of claim 9, wherein said cell line is a fibroblast cell line.
16. Expression vector comprising a mutated or attenuated virus and the isolated nucleic acid molecule of claim 1.
17. The expression vector of claim 16, wherein said virus is adenovirus or vaccinia virus.
18. The expression vector of claim 17, wherein said virus is vaccinia virus.
19. The expression vector of claim 17, wherein said virus is adenovirus.
20. Expression system useful in transfecting a cell, comprising (i) a first vector containing a nucleic acid molecule which codes for the isolated cancer associated antigen of claim 8 and (ii) a second vector selected from the group consisting of (a) a vector containing a nucleic acid molecule which codes for an MHC or HLA molecule which presents an antigen derived from said cancer associated antigen and (b) a vector containing a nucleic acid molecule which codes for an interleukin.
21. Isolated cancer associated antigen comprising the amino acid sequence encoded by nucleotides 287-3714 of SEQ ID NO: 1.

22. Immunogenic composition comprising the isolated antigen of claim 21, and a pharmaceutically acceptable adjuvant.

23. The immunogenic composition of claim 22, wherein said adjuvant is a cytokine, a saponin, or GM-CSF.

24. Immunogenic composition comprising at least one peptide consisting of an amino acid sequence of from 8 to 12 amino acids concatenated to each other in the isolated cancer associated antigen of claim 21, and a pharmaceutically acceptable adjuvant.

25. The immunogenic composition of claim 24, wherein said adjuvant is a saponin, a cytokine, or GM-CSF.

26. The immunogenic composition of claim 24, wherein said composition comprises a plurality of peptides which complex with a specific MHC molecule.

27. Isolated peptide derived from the amino acid sequence encoded by SEQ ID NO: 1, wherein said isolated peptide binds to an HLA molecule, is a nonamer, decamer or undecamer, and comprises the amino acid sequence of SEQ ID NO: 3, from one to three additional N-terminal amino acid, and up to four additional C terminal amino acids.

28. Immunogenic composition which comprises at least one expression vector which encodes for a peptide derived from the amino acid sequence encoded by SEQ ID NO: 1, and an adjuvant or carrier.

29. The immunogenic composition of claim 28, wherein said at least one expression vector codes for a plurality of peptides.

30. Vaccine useful in treating a subject afflicted with a cancerous condition comprising the isolated cell line of claim 11 and a pharmacologically acceptable adjuvant.

31. The vaccine of claim 30, wherein said cell line has been rendered non-proliferative.
32. The vaccine of claim 31, wherein said cell line is a human cell line.
33. A composition of matter useful in treating a cancerous condition comprising a non proliferative cell line having expressed on its surface a peptide derived from the amino acid sequence encoded by SEQ ID NO: 1.
34. The composition of matter of claim 33, wherein said cell line is a human cell line.
35. A composition of matter useful in treating a cancerous condition, comprising (i) a peptide derived from the amino acid sequence encoded by SEQ ID NO: 1, (ii) an MHC or HLA molecule, and (iii) a pharmaceutically acceptable carrier.
36. Isolated antibody which is specific for the antigen of claim 21.
37. The isolated antibody of claim 36, wherein said antibody is a monoclonal antibody.
38. Method for screening for cancer in a sample, comprising contacting said sample with a nucleic acid molecule which hybridizes to all or part of SEQ ID NO: 1, and determining hybridization as an indication of cancer cells in said sample.
39. A method for screening for cancer in a sample, comprising contacting said sample with the isolated antibody of claim 36, and determining binding of said antibody to a target as an indicator of cancer.
40. Method for diagnosing a cancerous condition in a subject, comprising contacting an immune reactive cell containing sample of said subject to a cell line transfected with the isolated nucleic acid molecule of claim 1, and determining interaction of said transfected cell line with said immunoreactive cell, said interaction being indicative of said cancer condition.

41. A method for determining regression, progression of onset of a cancerous condition comprising monitoring a sample from a patient with said cancerous condition for a parameter selected from the group consisting of (i) CT7 protein, (ii) a peptide derived from CT7 protein (iii) cytolytic T cells specific for said peptide and an MHC molecule with which it non-covalently complexes, and (iv) antibodies specific for said CT7 protein, wherein amount of said parameter is indicative of progression or regression or onset of said cancerous condition.

42. Method of claim 41, wherein said sample is a body fluid or exudate.

43. Method of claim 41, wherein said sample is a tissue.

44. Method of claim 41, comprising contacting said sample with an antibody which specifically binds with said protein or peptide.

45. Method of claim 44, wherein said antibody is labelled with a radioactive label or an enzyme.

46. Method of claim 44, wherein said antibody is a monoclonal antibody.

47. Method of claim 41, comprising amplifying RNA which codes for said protein.

48. Method of claim 47, wherein said amplifying comprises carrying out polymerase chain reaction.

49. Method of claim 41, comprising contacting said sample with a nucleic acid molecule which specifically hybridizes to a nucleic acid molecule which codes for or expresses said protein.

50. Method of claim 41, comprising assaying said sample for shed protein.

51. Method of claim 41, comprising assaying said sample for antibodies specific for said CT7 protein, by contacting said sample with CT7 protein.

52. Method for diagnosing a cancerous condition comprising assaying a sample taken from a subject for an immunoreactive cell specific for a peptide derived from CT7, complexed to an MHC molecule, presence of said immunoreactive cell being indicative of said cancerous condition.

53. An isolated nucleic acid molecule which encodes a protein and which has a complementary sequence which hybridizes, under stringent conditions, to at least one of the nucleotide sequences set forth at SEQ ID NO: 5, 6, 7 or 8.

54. The isolated nucleic acid molecule of claim 53, wherein said protein is the protein encoded by the nucleotide sequence of SEQ ID NO: 5, 6, 7 or 8.

55. The isolated nucleic acid molecule of claim 53, selected from the group consisting of nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO: 5, 6, 7 or 8.

56. Expression vector comprising the isolated nucleic acid molecule of claim 54, operably linked to a promoter.

57. Expression vector comprising the isolated nucleic acid molecule of claim 55, operably linked to a promoter.

58. Recombinant cell comprising the expression vector of claim 56.

59. Recombinant cell comprising the expression vector of claim 57.

60. Recombinant cell comprising the isolated nucleic acid molecule of claim 54.

61. Recombinant cell comprising the isolated nucleic acid molecule of claim 55.

62. Recombinant cell of claim 58, further comprising an expression vector which contains a nucleic acid molecule encoding a cytokine, operably linked to a promoter.

63. Recombinant cell of claim 59, further comprising an expression vector which contains a nucleic acid molecule encoding a cytokine, operably linked to a promoter.

64. Recombinant cell of claim 60, further comprising a nucleic acid molecule which encodes a cytokine.

65. Recombinant cell of claim 61, further comprising a nucleic acid molecule which encodes a cytokine.

66. The recombinant cell of claim 62, 63, 64, or 65, wherein said cytokine is interleukin.

67. The recombinant cell of claim 66, wherein said interleukin is 1L-2, 1L-4, or 1L-12.

68. The recombinant cell of claim 58, 59, 60, or 61, wherein said recombinant cell is a eukaryotic cell.

69. The recombinant cell of claim 68, which has been rendered non-proliferative.

70. The recombinant cell of claim 68, wherein said cell is a fibroblast.

71. Expression vector comprising a mutated or attenuated virus and the isolated nucleic acid molecule of claim 53, 54 or 55.

72. The expression vector of claim 71, wherein said virus is adenovirus, adeno associated virus, or vaccinia virus.

73. Expression system useful in making a recombinant cell, comprising:

(i) a first vector which encodes the protein encoded by the isolated nucleic acid molecule of claim 53, 54 or 55, and

(ii) a second vector which either (a) encodes an MHC or HLA molecule or (b) encodes an interleukin.

74. An isolated cancer associated antigen comprising the amino acid sequence encoded by SEQ ID NO: 5, 6, 7 or 8.

75. Composition comprising the isolated cancer associated antigen of claim 74, and a pharmaceutically acceptable adjuvant.
76. The composition of claim 75, wherein said adjuvant is a cytokine, a saponin, or GM-CSF.
77. Composition comprising at least one peptide consisting of an amino acid sequence of from 8 to 25 amino acids concatenated to each other in the isolated cancer associated antigen of claim 74, and a pharmaceutically acceptable adjuvant.
78. The composition of claim 77, wherein said adjuvant is a saponin, a cytokine, or GM-CSF.
79. The composition of claim 77, comprising a plurality of MHC binding peptides.
80. Composition comprising an expression vector which encodes at least one peptide consisting of an amino acid sequence of from 8 to 25 amino acids concatenated to each other in the isolated cancer associated antigen of claim 74, and pharmaceutically acceptable adjuvant.
81. The composition of claim 80, wherein said expression vector encodes a plurality of peptides.
82. Composition useful in treating a subject afflicted with a cancer, comprising the recombinant cell of claim 69 and a pharmacologically acceptable adjuvant.
83. The composition of claim 82, wherein said recombinant cell expresses an HLA or MHC molecule.
84. The composition of claim 82, wherein said recombinant cell is a human cell.
85. The composition of claim 77, further comprising at least one MHC or HLA molecule.

86. Isolated antibody which specifically binds to the isolated cancer associated antigen of claim 74.

87. The isolated antibody of claim 86, wherein said antibody is a monoclonal antibody.

88. A method for screening for possible presence of a pathological condition, comprising assaying a sample from a patient believed to have a pathological condition for antibodies specific to at least one of the cancer associated antigens encoded by SEQ ID NOS: 4, 5, 6, 7 or 8, presence of said antibodies being indicative of possible presence of said pathological condition.

89. The method of claim 88, wherein said pathological condition is cancer.

90. The method of claim 89, wherein said cancer is melanoma.

91. The method of claim 90, further comprising contacting said sample to purified cancer associated antigen encoded by SEQ ID NO: 4, 5, 6, 7 or 8.

92. A method for screening for possible presence of a pathological condition in a subject, comprising assaying a sample taken from said subject for expression of a nucleic acid molecule, the nucleotide sequence of which comprises SEQ ID NO: 5, 6, 7 or 8, expression of said nucleic acid molecule being indicative of possible presence of said pathological condition.

93. The method of claim 92, wherein said pathological condition is cancer.

94. The method of claim 92, comprising determining expression via polymerase chain reaction.

95. The method of claim 92, comprising determining expression by contacting said sample with at least one of SEQ ID NO: 11, 12, 13 or 14.



96. A method for determining regression, progression or onset of a cancerous condition comprising monitoring a sample from a patient with said cancerous condition for a parameter selected from the group consisting of (i) a cancer associated antigen encoded by SEQ ID NO: 3, 4, 5 or 6, (ii) a peptide derived from said cancer associated antigen, (iii) cytolytic T cells specific for said peptide and an MHC molecule with which it non-covalently complexes, and (iv) antibodies specific for said cancer associated antigen, wherein amount of said parameter is indicative of progression or regression or onset of said cancerous condition.

97. The method of claim 96, wherein said sample is a body fluid or exudate.

98. The method of claim 96, wherein said sample is a tissue.

99. The method of claim 96, comprising contacting said sample with an antibody which specifically binds with said protein or peptide.

100. The method of claim 99, wherein said antibody is labelled with a radioactive label or an enzyme.

101. The method of claim 99, wherein said antibody is a monoclonal antibody.

102. The method of claim 96, comprising amplifying RNA which codes for said protein.

103. The method of claim 102, wherein said amplifying comprises carrying out polymerase chain reaction.

104. The method of claim 96, comprising contacting said sample with a nucleic acid molecule which specifically hybridizes to a nucleic acid molecule which codes for or expresses said protein.

105. The method of claim 96, comprising assaying said sample for shed cancer associated antigen.

106. The method of claim 96, comprising assaying said sample for antibodies specific for said cancer associated antigen, by contacting said sample with said cancer associated antigen.

107. Method for screening for a cancerous condition comprising assaying a sample taken from a subject for an immunoreactive cell specific for a peptide derived from a cancer associated antigen encoded by SEQ ID NO: 4, 5, 6, 7 or 8 complexed to an MHC molecule, presence of said immunoreactive cell being indicative of said cancerous condition.

108. An isolated nucleic acid molecule consisting of a nucleotide sequence defined by SEQ ID NO: 9, 10, 11, 12, 13 or 14.

109. Kit useful in determining expression of a cancer associated antigen, comprising a separate portion of each of (i) the nucleotide sequences defined by SEQ ID NOS: 9 and 10, (ii) the nucleotide sequences defined by SEQ ID NOS: 11 and 12, and (iii) the nucleotide sequences defined by SEQ ID NOS: 13 and 14.

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<120> Isolated Nucleic Acid Molecules Encoding Cancer Associated Antigen, The  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/05766

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(6) : G01N 33/574, 33/48; C12Q 1/68 US CL : 435/7.23, 6; 436/64 According to International Patent Classification (IPC) or to both national classification and IPC		
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/7.23, 6; 436/64 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) APS, DIALOG		
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TURECI, O. et al. Expression of SSX Genes in Human Tumors International Journal of Cancer. 1998, Vol. 77, No. 1, pages 19-23, especially page 19.	41-52
A	GURE, A.O. et al. SSX: A Multigene Family with Several Members Transcribed in Normal Testis and Human Cancer. International Journal of Cancer. 1997, Vol. 72, No. 6, pages 965-971, especially page 965.	41-52
A	CHEN, et al. A Testicular Antigen Aberrantly Ex[pressed in Human Cancers Detected by Autologous Antibody Screening. Proceedings of the National Academy of Science. March 1997, Vol. 94, No. 5, pages 1914-1918, especially page 1914.	41-52
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A' document defining the general state of the art which is not considered to be of particular relevance	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E' earlier document published on or after the international filing date	*Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G	document member of the same patent family
*O' document referring to an oral disclosure, use, exhibition or other means		
*P' document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search	Date of mailing of the international search report	
21 JULY 1999	11 AUG 1999	
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Yvonne EYLER</i> YVONNE EYLER Telephone No. (703) 308-0196	

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/05766

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	CHEN, et al. Identification of Multiple Cancer/Testis Antigens by Allogeneic Antibody Screening of a Melanoma Cell Line Library" Proceedings of the National Academy of Science. June 1998, Vol. 95, No. 12, pages 6919-6923, especially page 6919.	41-52
P,Y	UGUR, S. et al. Expression of Multiple Cancer/Testis (CT) Antigens in Breast Cancer and Melanoma: Basis for Polyvalent CT Vaccine Strategies. International Journal of Cancer. 1998, Vol. 78, No. 3, pages 387-389, especially page 387.	41-52

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